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RECEPTOR BINDING AND MEMBRANE TRANSPORT OF BOTULINUM TOXINS

ANNUAL REPORT

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This study centers upon the development of a model membrane system for the study of the membrane-active protein toxin, botulinum. The model membrane system requires 1) that the membrane retains impermeability to ions and small molecules during an acid shock, 2) that large numbers of botulinum toxin may be recovered from the membrane after any membranolytic activity has been produced from the toxin by acid shock.

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Introduction

The study of the mechanism of action of botulinum toxin (botox) would be greatly aided by defining a model system suitable for study which mimics the action of the toxin molecule in vivo. Towards this goal we have endeavored to define a model system of study for botox. It has become apparent that different toxin molecules, such as diphtheria or bacteriocins, contain some basic features which are similar for almost all of these types of molecules. Since these molecules also share a similar function (i.e., to bind to a specific receptor, translocate a portion of the molecule into the target, and thereby destroy the target cell) it is not difficult to imagine that a similar structure exists between these molecules from diverse sources.

Recent reports on the ability of the botox molecule in supported planar bilayer membranes have concluded that a low pH value of the medium allows the molecule to produce ion channels in the membrane (1,2). This is also similar to the known channel forming ability of other toxins, such as diphtheria (3) and colicin E1 (4). The production of channels in supported planar bilayers is a system which serves well to characterize these ion disturbances in the membrane, and is one of the most sensitive assays in that the current produced by a single molecule can be measured (5). This sensitivity is an obstacle however when one wishes to recover and study the toxin molecule from the assay chamber after channel formation in that so few molecules are recovered. One system that allows the study of both membrane activity and yields large numbers of molecules for recovery is the system of artificial liposomes. Again, for other toxin molecules, investigations on the orientation of the molecule in the bilayer have utilized similar vesicle systems.

For the present study it was determined to produce a liposomal system of study for the botox toxin. This system would ideally provide i) the expression of membranolytic activity by the toxin, ii) recovery of the molecule before and after the "membrane activity" to investigate the changes that have occurred in the molecule, and iii) a vesicular system which would withstand the effects of triggering membrane activity (i.e., pH drop to 4) in the absence of toxin. In addition the system must not allow the molecule to express membranolytic activity prior to the pH drop. From such a system it is anticipated that both the requirements for membrane binding and channel activity expression can be determined. This can be used to determine which portions of the molecule are embedded in the membrane, with the ultimate goals to define the orientation of the toxin in the membrane and thereby ascertain which portions of the molecule are best suited for targeting by antibodies or other inhibitors.

Methods

Supplies. Sources for all chemicals and reagents for buffers and solutions unless otherwise stated were purchased from Fisher Scientific. Dyes for the fluorescence studies from Molecular Probes, Inc. Lipids were obtained from Avanti Polar Lipids, and all electrophoretic chemicals were purchased from Serva. Botulinum toxin (Type B) was obtained from Calbiochem.

Lipid Vesicle Formation. Large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were formed as described in references (6) and (7).

Acid Shock Procedure. Vesicles in 10 mM Imidazole buffer containing 150 nM NaCl, pH 7 (10 µg lipid/mL) were subjected to a drop in pH by the addition of 10 µL of a 10% stock solution of succinic acid at pH 3.9. After 1 min the entire mixture was diluted into 3 mL of buffer at pH 7.4.

Fluorescence Measurements. Determination of release of the dye carboxyfluorescein (CF) from lipid vesicles was performed by forming the vesicles in the presence of a self-quenching concentration of the dye molecule (100 mM). The free dye molecules were separated from the internalized dye and liposomes by either passing the mixture over a Sephadex G-100 column (collecting the void volume fractions of the column for the vesicles) or by repeated centrifugations (at least 3 times) with resuspension into dye-free buffer. The vesicles were then used within 4 hours for all assays of toxin activity. Measurement of dye release was performed on an Aminco Bowman Spectrophotofluorometer, adapted with a thermostatted, stirred cuvette, as described in reference (7).

For the measurement of potassium diffusion potentials the dye ANS (8-anilino-1-naphthalene sulphonate) was utilized as an indicator (8). Measurements were performed essentially by the method described in (3) with vesicles containing KCl rather than NaCl as described above, and were diluted into potassium-free buffer which contained choline chloride in place of KCl or NaCl in the cuvette of the fluorometer at 37°C.

Results

The requirements for an assay system which could withstand a pH shock (i.e., from pH 7 to pH 4 for 1 min, returning to pH 7) led us to look for a liposomal system which would allow a rapid screening of membrane bilayer integrity. It was determined that two separate assay systems would also be of advantage in order to confirm the results of a single assay system. To meet both the above terms two separate fluorescence assays were utilized. The first relies upon the self-quenching ability of the dye carboxyfluorescein (CF). At high concentrations (above 16 mM) the fluorescence of the dye is low, once the concentration is lowered the fluorescence dramatically increases. Using vesicles containing CF at 100 mM, any release of dye into the surrounding medium (which essentially dilutes the concentration over 200 times) results in a dramatic increase in signal. Figure 1 demonstrates the increase in fluorescence signal upon addition of a detergent to vesicles containing 100 mM CF. This system was used to determine the ability of liposomes of various composition to withstand an acid shock (lowering of pH for 1 min). Any increase in signal after returning the vesicles to pH 7 would indicate that the bilayer integrity of the vesicles had been compromised. Table 1 summarizes the results of the acid shock procedure on a variety of liposomal compositions, which includes vesicles formed in the presence of ganglioside GM_1, reported as a possible receptor molecule for botox in vivo (9). It can be seen that of the vesicle compositions tested that some difference is seen between a pH drop of two units (to pH 5) versus three pH units (to pH 4). Since maximal channel activity for this toxin (10) and others (3) has been shown to be nearer to pH 4 it was determined that only those that could withstand the pH 4 shock could be utilized further. From these studies it was determined that vesicles composed of 1) 100% Egg PC, 2) 99% Egg PC, 1% ganglioside GM_1, 3) 50% DPPC/50% DOPC, 4) 45% DPPC/45% DOPC/5% cholesterol, and those composed as 3) and 4) with 1% added ganglioside could be utilized for further studies.

These selected vesicle compositions were then assayed for the ability to develop potassium diffusion potentials and monitoring of this potential by the second fluorescence assay utilizing the potential sensitive dye ANS. Figure 2 demonstrates the fluorescence assay showing how the addition of the potassium carrier valinomycin can induce a change in the fluorescence of this dye. As the positively charged potassium ions escape the vesicle (the outside medium containing 100 times less potassium) without a concomitant loss of negative charges (or influx of positive charges) a membrane potential (trans-negative) develops to a size dependent upon the ratio of K+ in/K+ out (i.e., according to the Nernst equation (11)). The negatively charged dye ANS is then repelled from the bilayer due to the internal negative charge, and the less hydrophobic environment of the aqueous medium results in a decrease in fluorescent yield. Table 2 shows how the six liposome types described above respond to the induction of membrane potential after an acid shock. For all of these types lacking the ganglioside GM_1 no loss of membrane integrity was detected. For the vesicles containing the ganglioside a slight leak is seen which might be due to the presence of the membrane potential inducing either the ganglioside or some contaminant in the ganglioside preparation to shuttle K+ ions (or other ions) across the membrane.

For directly examining the membranolytic effect of botulinum toxin, liposomes were subjected to an acid shock in the presence of toxin molecule, a control protein bovine serum albumin (BSA), or no added protein. Table 3 shows that the botox molecule is able to induce a breakdown of permeability according

to both dye assays (CF and ANS) with the aid of an acid shock (pH 4 for 1 min). It was also seen that the vesicles containing the ganglioside were subject to a breakdown in the membrane barrier when acid shocked in the presence of BSA, indicating that future studies utilizing this agent in the formation of vesicles would not be able to distinguish between botox-induced channels from non-specific leaks caused by the presence of any control protein not known to express channel activity (i.e., BSA). The nature of this non-specific leak is not known, but was also seen when tested with other proteins such as lysozyme, human complement component C8, and trypsin (data not shown).

Discussion

The ability of botulinum toxin (botox) to cause membrane damage has been established through the use of planar membrane experiments (1,2) to be pH-dependent, and that as the pH decreases to 4 this channel activity increases. As many toxins, viruses, and other molecules gain access to the interior of the cell via endocytic vesicles that become acidified triggering release into the cell (9,12) this phenomenon is consistent with a similar mechanism for botox. The present study has sought to define conditions that will enable us to probe the molecule after it has performed any membranolytic activity, presumably by inserting one or more portions of the protein into the hydrophobic interior of the bilayer. Future studies will focus on defining which portions of the botox molecule remain exposed on the surface (through the use of proteases and antibodies) and which portion(s) are buried into the hydrophobic core of the bilayer of the membrane (through the use of a membrane-restricted photolabel). The definition of desired vesicle characteristics for use as targets of attack by botox are most important to gain an understanding of the mechanism of action of the toxin. Our results would argue that studies utilizing the ganglioside GM_1 may confuse the results of future studies to determine the topography of the toxin embedded in or in the process of it's reported channel activity. Although gangliosides may be important in directing the toxin to a target cell, the actual lethal event may be dependent upon crossing a membrane barrier, dissipating a chemical gradient, or introducing an enzymatically lethal entity into the interior of a target cell. It is of interest to note in this context that the channel activity of the toxin molecule was <u>not</u> dependent upon gangliosides, and that botox added to a chamber (2-4 mL volume) could express activity on a supported bilayer of that large chamber (1).

Table 1. Stability of Artificial Toxin Targets Subjected to pH Shock.

Composition (%)	% Dye Release	
	pH 5	pH 4
Asolectin	15 ± 8	66 ± 14
Asolectin/GM1 (99/1)	23 ± 8	46 ± 12
Asolectin/CHOL (95/5)	7 ± 4	45 ± 8
DMPC (100)	47 ± 11	78 ± 6
DMPC/DPPC (50/50)	18 ± 6	34 ± 7
DMPC/DPPC/CHOL (48/47/5)	14 ± 5	33 ± 10
Egg PC (100)	6 ± 3	8 ± 2
Egg PC/GM1 (99/1)	7 ± 3	8 ± 4
DPPC (100)	5 ± 2	7 ± 4
DPPC/DOPC (50/50)	4 ± 2	4 ± 3
DPPC/DOPC/GM1 (50/49/1)	8 ± 3	18 ± 6
DPPC/DOPC/CHOL (48/47/5)	2 ± 1	2 ± 2
DPPC/DOPC/CHOL/GM1 (48/46/5/1)	4 ± 2	12 ± 5

GM1=ganglioside GM_1; CHOL= cholesterol;
PCs DM,DP,and DO= dimyristoyl, dipalmitoyl, and dioleoyl phosphatidylcholines, respectfully.
% Dye release= relative to detergent (triton x-100) ± SEM.

Table 2. Inhibition of Valinomycin-induced Membrane Potential by Acid Shock in Liposomes.

Composition (%)	% Inhibition
Egg PC (100)	10 ± 4
Egg PC/GM1 (99/1)	33 ± 8
DPPC/DOPC (50/50)	5 ± 3
DPPC/DOPC/GM1 (50/49/1)	25 ± 5
DPPC/DOPC/CHOL (48/47/5)	3 ± 1
DPPC/DOPC/CHOL/GM1 (48/46/5/1)	16 ± 4

GM1= ganglioside GM_1; CHOL= cholesterol;
PCs DM,DP,and DO= dimyristoyl, dipalmitoyl, and dioleoyl phosphatidylcholines, respectfully.
% Inhibition= Inhibition of ANS signal decrease, as in Methods, expressed ± SEM.

Table 3. pH-Induced Increase in Permeability of Liposomes by Botulinum Toxin and Bovine Serum Albumin (BSA).

Composition (% as in Table 2)	CF Release	ANS Inhibition
Egg PC + Botox	95 ± 9	87 ± 10
" + BSA	9 ± 6	8 ± 4
Egg PC/GM1 + Botox	99 ± 3	90 ± 12
" / " + BSA	94 ± 10	95 ± 9
DPPC + Botox	5 ± 4	8 ± 6
" + BSA	3 ± 2	4 ± 2
DPPC/DOPC + Botox	75 ± 8	87 ± 11
" / " + BSA	9 ±	46 ± 5
DPPC/DOPC/GM1 + Botox	88 ± 5	91 ± 13
" / " / " + BSA	73 ± 15	85 ± 14
DPPC/DOPC/CHOL + Botox	101 ± 12	89 ± 13
" / " / " + BSA	5 ± 2	3 ± 3
DPPC/DOPC/CHOL/GM1 + Botox	98 ± 14	78 ± 12
" / " / " / " + BSA	57 ± 8	67 ± 10

CF Release, as in Table 1; ANS Inhibition, as in Table 2. (All as ± SEM).

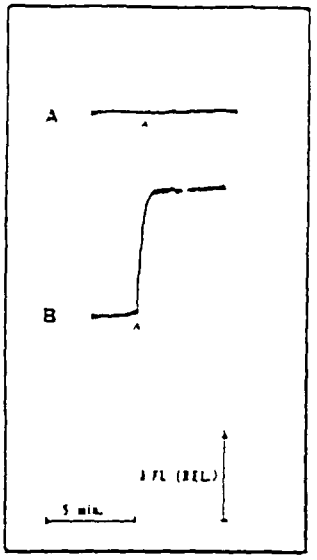

Figure 1. Release of entrapped Carboxyfluorescein (CF) from Asolectin Vesicles. Vesicles (1mg/mL) containing 100 mM CF, 10 mM Tris,150 mM NaCl (pH 7.2) were diluted 3000-fold into CF free buffer. At the indicated arrow Triton X-100 was added to a concentration of 0.1%. Fluorescence was monitored at 520 ± 4nm (excit = 480 ±4 nm). T=23°C, cuvette volume = 300 µL.

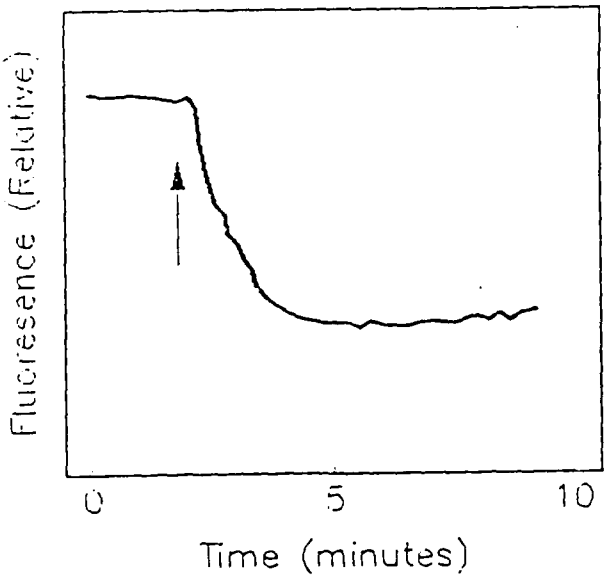

Figure 2. Fluorescence of the dye ANS in response to a valinomycin-induced potassium-diffusion potential in Asolectin vesicles. The dye ANS (8-anilino-1-naphthalene sulphonate) was utilized as an indicator (8). Measurements were performed essentially by the method described in (3) with vesicles containing KCl rather than NaCl as described in Fig. 1, and were diluted into potassium-free buffer which contained choline chloride in place of KCl or NaCl in the cuvette of the fluorometer at 37°C. At the indicated arrow, valinomycin was added to 10 nM.

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